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14. ABSTRACT: The proteasome inhibitor bortezomib (PS-341, Velcade) synergizes with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) acts via a p21-dependent mechanism to induce high levels of apoptosis in prostate cancer cells. Our further investigation into the molecular mechanisms underlying the effects of bortezomib implicated endoplasmic reticular (ER) stress in its anti-tumoral effects. These effects also provide us with a molecular mechanism to explain the observed anti-angiogenic effects of bortezomib in prostate cancer cells. We have generated luciferase-transduced variants of our human prostate cancer cell lines in order to use them to generate orthotopic tumors in nude mice that can be imaged non-invasively. We plan to use these models in the coming 6-12 months to test the toxicity and anti-tumor efficacy of combination therapy with bortezomib plus TRAIL in vivo. Preliminary toxicity studies confirmed that mice tolerate daily therapy with recombinant TRAIL plus biweekly therapy with bortezomib (at its MTD) very well.					
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INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer-related death. Although the use of PSA screening has led to more frequent detection of early disease that can be managed with surgery and/or hormonal therapy (androgen ablation), the development of androgen-independent disease is still a major clinical challenge, and no current therapeutic approach has demonstrated efficacy in this setting. Therefore, there is a critical need for novel therapeutic approaches that will be effective in androgen-independent prostate cancer. Current research is aimed at defining the biological mechanisms underlying androgen independence with the expectation that this information will identify new targets for therapeutic intervention.

We have been studying the effects of proteasome inhibitors in preclinical models of human prostate cancer for almost a decade. Our early work demonstrated that these agents are capable of inhibiting the inflammation-associated transcription factor, NF κ B, and bypassing some of the molecular mechanisms implicated in androgen-independent tumor cell survival (for example, overexpression of BCL-2) (1, 2). These observations prompted the initiation of the first-in-man Phase I clinical trial of one of these agents (PS-341, also known as bortezomib or Velcade) in men with androgen-independent prostate cancer, where it displayed promising clinical activity and produced biological effects consistent with NF κ B inhibition (suppression of IL-6 production) (3, 4). Based on these promising data a Phase II trial of bortezomib plus mitoxantrone was recently performed at our institution in men with androgen-independent prostate cancer, where even better effects were observed (A. Siefker-Radtke, personal communication). We also demonstrated that proteasome inhibitors block tumor angiogenesis by suppressing VEGF expression (5). As will be discussed below, we have now identified the molecular mechanism underlying this effect on VEGF expression, and it does not appear to involve NF κ B inhibition (Zhu et al, in preparation). Finally, in studies that served as the basis for the present project, we discovered that proteasome inhibitors synergize with the pro-apoptotic cytokine, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), to induce rapid and extensive apoptosis in all human prostate cancer cells and many other solid tumor cell types (6-8). Analysis of the molecular mechanisms involved strongly suggested again that NF κ B inhibition was not involved in TRAIL sensitization, contrary to our expectations. Rather, TRAIL sensitization appeared to be related to PS-341-induced cell cycle arrest, and more specifically to p21 accumulation, since chemical inhibitors of cyclin-dependent kinases mimicked the effects of bortezomib, and siRNA-mediated knockdown of p21 attenuated cell death (7). More recent work by other groups has confirmed that p21 promotes TRAIL sensitization (9), but other groups have argued that p21 inhibits cell death (10).

However, in work supported by this grant, we now have data that suggest that the effects of p21 on TRAIL may be unrelated to cell cycle arrest. First, direct transfection with p21 did not enhance TRAIL sensitivity in LNCaP-Pro5 cells, even though it blocked cell cycle progression (K. Zhu, unpublished). Furthermore, siRNA-mediated silencing of cdk1, cdk2, or both kinases also failed to promote TRAIL sensitivity in the cells (K. Zhu, unpublished). We now suspect that bortezomib promotes TRAIL sensitivity by activating an intracellular program known as the unfolded protein response (UPR), a coordinated mechanism that promotes cell survival and/or death in response to endoplasmic reticular (ER) stress (11). A central component of the UPR is the suppression of global protein synthesis (11), and inhibitors of protein or mRNA synthesis are known to reverse cellular resistance to death receptor-mediated apoptosis in other model systems (12). We also suspect that ER stress mediates the effects of bortezomib on cellular DR5 expression (Task 2) via induction of the transcription factor, GADD153 (also known as CHOP) (13). Finally, the UPR may also contribute to the inhibition of VEGF production observed in prostate cancer cells exposed to proteasome inhibitors (see below). Thus, our recent studies have prompted us to redirect focus away from the cell cycle effects of bortezomib to its effects on the UPR and ER stress. We suspect that we may be able to reinterpret the results we obtained with cdk inhibitors or p21 silencing within this new context.

BODY

Statement of Work

Task 1: To examine the roles of cell cycle arrest and changes in FADD phosphorylation and localization in bortezomib-mediated TRAIL sensitization. (Months 1-24)

Task 2: To evaluate the importance of bortezomib-induced upregulation of DR5 and define the molecular mechanisms involved. (Months 12-30)

Task 3: To assess the efficacy and potential toxicity of combined therapy with bortezomib plus TRAIL in orthotopic human prostate cancer xenografts. (Months 1-36)

Progress

Task 1: We have obtained a second clinically relevant proteasome inhibitor (NPI-0052) from Nereus Pharmaceuticals, Inc (San Diego, CA). The compound has several features that may make it superior to bortezomib, including its potency, its distinct spectrum of effects on the 3 proteolytic activities of the proteasome, its oral availability, and its irreversibility (bortezomib is a reversible inhibitor) (14, 15). Like bortezomib, NPI-0052 inhibits cell cycle progression and stabilizes p53 and p21, and it is a potent TRAIL-sensitizing agent (K. Zhu, A. Metwalli, unpublished observations).

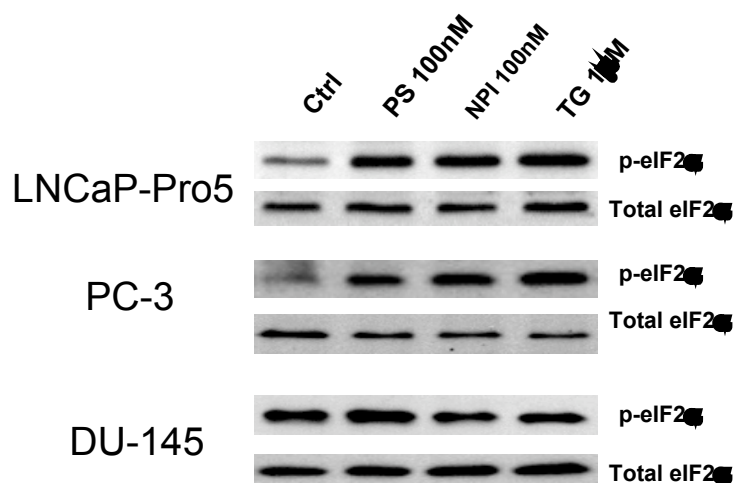
As discussed above, we have obtained good evidence that p21-mediated cell cycle arrest is insufficient to promote TRAIL sensitivity in human prostate cancer cells. Furthermore, in parallel studies we used phospho-specific antibodies to determine the role of FADD phosphorylation (S194) (16) in bortezomib-induced TRAIL sensitization. We consistently observed a very modest increase in FADD phosphorylation in cells exposed to either bortezomib alone or bortezomib plus TRAIL (S. Williams, K. Zhu, D.J. McConkey, unpublished observations).

The proteasome plays an essential role in mediating the degradation of misfolded, oxidized, or aggregated proteins (17). When this material accumulates excessively, it activates a coordinated cellular response known as the unfolded protein response (UPR) (17). At the core of the UPR is a protein kinase known as PKR-related ER kinase (PERK) that phosphorylates the eIF2 α translational initiation factor, thereby suppressing the translation of most transcripts and reducing protein synthetic burden (11, 17). Because inhibitors of protein or mRNA synthesis are known to promote TRAIL-mediated apoptosis (9), proteasome inhibitor-induced activation of the UPR could explain the TRAIL sensitization we observe in human prostate cancer cells exposed to bortezomib or NPI-0052 plus TRAIL.

To determine whether or not proteasome inhibitors stimulate the UPR in prostate cancer cells, we exposed them to bortezomib or NPI-0052 and measured eIF2 α phosphorylation by immunoblotting. Both drugs stimulated strong increases in LNCaP-Pro5 and PC-3 but not DU-145 cells (Figure 1).

Figure 1: Effects of proteasome inhibitors on eIF2 α phosphorylation.

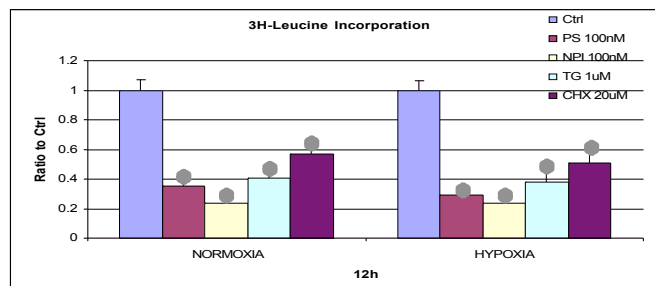
Cells were incubated for 4 h in the absence (control, Ctrl) or presence of 100 nM PS-341 (PS), 100 nM NPI-0052 (NPI), or 1 mM thapsigargin (TG, positive control), and eIF2 α phosphorylation was measured by immunoblotting using a phospho-specific antibody. Equivalent volumes of the lysates were run on separate gels, and total eIF2 α was also measured by immunoblotting (loading controls). Results are representative of those obtained in 3 separate experiments.



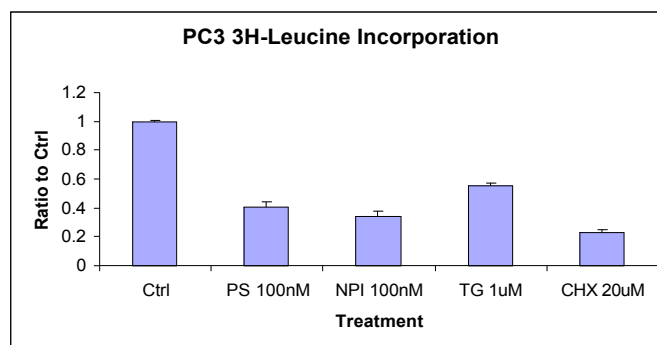
To determine whether or not these changes in eIF2 α phosphorylation resulted in an inhibition of global protein synthesis, we exposed LNCaP-Pro5 or PC-3 cells to PS-341, NPI-0052, thapsigargin, or cycloheximide (positive control) and measured protein synthesis by 3 H-leucine incorporation. As expected, all of these agents inhibited translation in the LNCaP-Pro5 and PC-3 cells (Figure 2) but not in the DU-145 cells (data not shown).

Figure 2: Effects of proteasome inhibitors on translation. Cells were incubated for 12 h in the absence or presence of the indicated compounds, and global protein synthesis was measured by 3 H-leucine incorporation. Similar results were observed at 4 h. Mean \pm SEM. n = 8.

LNCaP

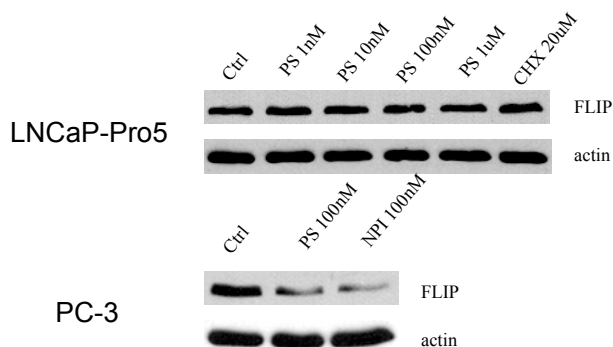


PC-3



Previous work by Debatin's laboratory showed that cycloheximide sensitizes tumor cells to TRAIL by downregulating expression of the decoy caspase and TRAIL inhibitor, c-FLIP (9). Other investigators concluded that c-FLIP is a major determinant of TRAIL resistance in human prostate cancer cells (18). Therefore, we examined the effects of proteasome inhibitors on c-FLIP expression in the two cell lines that displayed strong reductions in protein synthesis (LNCaP-PRO5 and PC-3). Interestingly, neither PS-341 nor cycloheximide downregulated c-FLIP expression in the LNCaP-Pro5 cells, whereas both proteasome inhibitors rapidly downregulated c-FLIP in PC-3 cells (Figure 3). Therefore, c-FLIP appears to be more stable in the LNCaP-Pro5 cells than it is in the PC-3 cells and is a potential mediator of bortezomib-mediated TRAIL sensitization in the latter.

Figure 3: Effects of proteasome inhibitors on expression of the TRAIL inhibitor, c-FLIP. Cells were incubated for 24 h (LNCaP-Pro5) or 6 h (PC-3) in the absence or presence of the indicated compounds, and c-FLIP levels were measured by immunoblotting. Blots were then stripped and re-probed with an anti-actin antibody to confirm equal protein loading.



As discussed above, we previously showed that proteasome inhibitors block VEGF production in human prostate cancer cells (5). Paradoxically, VEGF's major regulator (hypoxia inducible factor-1a, or HIF-1a) is maintained at low levels in cells growing under normoxia (atmospheric oxygen levels) by the tumor suppressor protein, VHL, which targets it for degradation via the proteasome (19). Thus, one would expect that proteasome inhibitors would **upregulate** HIF-1 α levels under normoxic conditions, potentially leading to increased VEGF production.

We therefore investigated the effects of proteasome inhibitors on HIF-1 α protein expression and activity in human prostate cancer cells. We incubated cells under conditions of low oxygen (0.2%) in a hypoxia chamber in the absence or presence of proteasome inhibitors and measured HIF-1 α levels by immunoblotting. We also incubated cells in cobalt chloride (to mimic hypoxia) in the absence or presence of bortezomib or NPI-0052. Strikingly, both drugs blocked HIF-1 α accumulation (Figure 4) and downregulated HIF-1 α -dependent promoter activity, measured in cells transfected with a HIF-1 α reporter construct (Figure 5). Interestingly, these effects correlated inversely with the effects of proteasome inhibitors on eIF2 α phosphorylation, in that proteasome inhibitors actually promoted HIF-1 α accumulation in cells that did not display eIF2 α phosphorylation in response to proteasome inhibition (i.e., DU145 and 253J B-V cells, Figure 4 and data not shown). Importantly, real time quantitative PCR demonstrated that the proteasome inhibitors had no effect on HIF-1 α mRNA levels, demonstrating that the effects occurred post-transcriptionally. Thus, we suspect that the inhibition of translation induced by proteasome inhibitors is involved in the downregulation of HIF-1 α expression observed. We have obtained preliminary evidence that HIF-1 α is degraded via autophagy in the prostate cancer cells exposed to proteasome inhibitors (K. Zhu, unpublished observations).

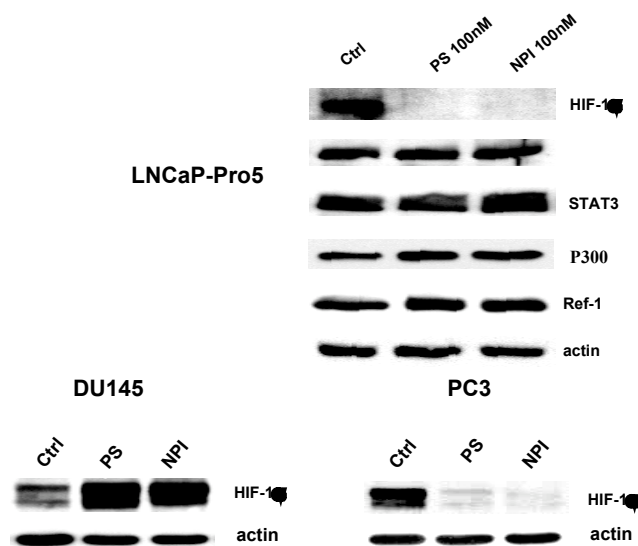


Figure 4: Effects of proteasome inhibitors on hypoxia-induced HIF-1 α accumulation. Note that HIF-1 α was undetectable in cells exposed to normoxia at this level of immunoblotting sensitivity. Indistinguishable results were obtained in cells exposed to cobalt chloride.

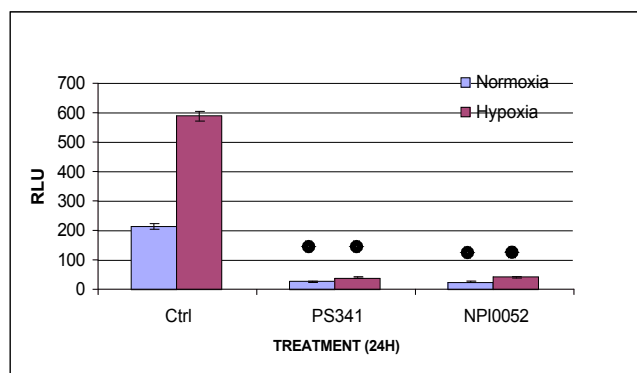


Figure 5: Effects of proteasome inhibitors on HIF-1 α promoter activity (LNCaP-Pro5 cells). Cells were transiently transfected with a construct encoding firefly luciferase under the control of a synthetic hypoxia response element.

Plans for the coming year: We have obtained constructs encoding mutant forms of eIF2 α (yeast and human) that cannot be phosphorylated by PERK. We will transfect or transduce LNCaP-Pro5 and PC-3 cells with these constructs to see if it can rescue translation in cells exposed to proteasome inhibitors. We will then investigate whether or not the mutant constructs attenuate proteasome inhibitor-mediated TRAIL sensitization,

thereby directly testing our hypothesis that activation of this component of the UPR is responsible for the effects observed. We will also determine whether or not the dominant active eIF2 α restores HIF-1 α levels in the cells. We will also investigate the potential effects of p21 silencing on proteasome inhibitor-induced activation of the UPR. Finally, we will investigate whether or not proteasome inhibitors induced molecular correlates of the UPR in peripheral blood mononuclear cells and primary tumor biopsies obtained from patients with androgen-independent prostate cancer who were enrolled in our Phase II trial of bortezomib plus mitoxantrone. We will correlate the levels of eIF2 α phosphorylation observed with the extent of 20S proteasome inhibition as measured using a fluorescent peptide substrate.

Task 2: Several transcription factors are upregulated by the UPR (11, 17). Of these, GADD153/CHOP has been most consistently implicated in cell death (11). We performed preliminary experiments with bortezomib and several other agents that activate the UPR in prostate cancer cells via independent mechanisms, and found that they all increase surface DR5 expression (K. Zhu, unpublished observations), consistent with observations made by another group with prostate cancer cells (20). Another group implicated CHOP in ER stress-mediated upregulation of DR5 in ovarian cancer cells (13).

We have obtained agonistic anti-DR4 and –DR5 antibodies from Human Genome Sciences, Inc (Rockville, MD). These reagents will enable us to determine whether or not the effects of proteasome inhibitors on DR5 expression render the cells especially sensitive to agonists of DR5.

Plans for the coming year: We will characterize the effects of proteasome inhibitors and other inducers of ER stress on CHOP expression in LNCaP-Pro5, PC-3, and DU145 cells. We expect that all of the agents will upregulate DR5 in the LNCaP-Pro5 and PC-3 cells because they appear to activate the UPR, whereas we do not expect proteasome inhibitors to do the same in DU145 cells (which display a defective UPR). We will use siRNA to knock down CHOP expression in LNCaP-Pro5 cells and investigate whether or not CHOP knockdown abolishes proteasome inhibitor-induced upregulation of DR5. We will also silence DR5 expression to determine whether or not the upregulation of DR5 is functionally important for TRAIL sensitization. Finally, we will compare the effects of proteasome inhibitors and other ER stress inducers on apoptosis induced by anti-DR4 and anti-DR5 antibodies. We would expect that cells will be especially sensitive to the latter, which could have important implications for future combination studies with these agents.

Task 3: We have performed preliminary toxicity studies in mice. We treated mice daily with recombinant human TRAIL (prepared in the laboratory)(10 mg/kg, 5x/week), biweekly with bortezomib (1 mg/kg), or both drugs for a total of 3 weeks. All of the mice (5 per group) survived therapy and displayed minimal weight loss. Therefore, it appears that a biologically effective dose of TRAIL can be administered with an MTD dose of bortezomib without excessive toxicity.

In preparation for our xenograft studies, we have stably transduced LNCaP-Pro5 and PC-3M cells with a lentiviral luciferase construct. This will enable us to quantify orthotopic tumor burden as we have done previously in human pancreatic tumor xenografts (8). We have confirmed that TRAIL, proteasome inhibitors, and combinations of them have similar effects in the transduced cells to those observed in the parental lines.

Plans for the coming year: We will implant the transduced cells orthotopically in nude mice and determine their tumorigenicity. We will “recycle” the cell lines through the orthotopic site if necessary to optimize tumor growth (21). We will then treat established orthotopic tumors with TRAIL, bortezomib, NPI-0052, or combinations of them to determine the effects of combination therapy on growth. Once the effects of these agents on prostate tumors have been determined we will investigate whether or not combination therapy inhibits the growth of tumors growing in the bone.

We also plan to develop cells transduced with reporter constructs that are driven by one or more of the transcription factors that are activated during the UPR (i.e., CHOP – see above). These cells could be used to non-invasively confirm that our proteasome inhibitors have the expected effects on the UPR in vivo.

KEY RESEARCH ACCOMPLISHMENTS

- Showed for the first time that proteasome inhibitors activate the UPR in some (but not all) human prostate cancer cells.
- Showed that proteasome inhibitors downregulate HIF-1 α in the same cells
- Obtained preliminary evidence that c-FLIP downregulation is important for TRAIL sensitization in PC-3 cells
- Obtained preliminary evidence that proteasome inhibitors activate autophagy in prostate cancer cells, and autophagy mediates the elimination of HIF-1 α protein in the cells
- Obtained preliminary evidence that ER stress upregulates DR5 expression in prostate cancer cells
- Determined that mice tolerate therapy with proteasome inhibitors plus TRAIL

REPORTABLE OUTCOMES

- Manuscript describing the effects of proteasome inhibitors on the UPR is in preparation
- Generated LNCaP-Pro5 and PC-3M cells stably transduced with luciferase

CONCLUSIONS

- Proteasome inhibitors induce ER stress in human prostate cancer cells
- The effects of proteasome inhibitors on specific components of the UPR (eIF2 α phosphorylation in particular) are heterogeneous and may correlate with effects on DR5 and HIF-1 α expression
- Downregulation of c-FLIP may contribute to proteasome inhibitor-mediated TRAIL sensitization in some (but not all) cells
- Combination therapy with proteasome inhibitors plus TRAIL is well tolerated in mice

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